Medroxyprogesterone acetate impairs human dendritic cell activation and function

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STUDY QUESTION: Does medroxyprogesterone acetate (MPA) impair human dendritic cell (DC) activation and function?

SUMMARY ANSWER: In vitro MPA treatment suppressed expression of CD40 and CD80 by human primary DCs responding to Toll-like receptor 3 (TLR3) agonist stimulation (i.e. DC activation). Moreover, this MPA-mediated decrease in CD40 expression impaired DC capacity to stimulate T cell proliferation (i.e. DC function).

WHAT IS KNOWN ALREADY: MPA is the active molecule in Depo-Provera® (DMPA), a commonly used injectable hormonal contraceptive (HC). Although DMPA treatment of mice prior to viral mucosal tissue infection impaired the capacity of DCs to up-regulate CD40 and CD80 and prime virus-specific T cell proliferation, neither DC activation marker expression nor the ability of DCs to promote T cell proliferation were affected by in vitro progesterone treatment of human DCs generated from peripheral blood monocytes.

STUDY DESIGN, SIZE, DURATION: This cross-sectional study examined MPA-mediated effects on the activation and function of human primary untouched peripheral blood DCs.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Human DCs isolated from peripheral blood mononuclear cells by negative immunomagnetic selection were incubated for 24 h with various concentrations of MPA. After an additional 24 h incubation with the TLR3 agonist polyinosinic:polycytidylic acid (poly I:C), flow cytometry was used to evaluate DC phenotype (i.e. expression of CD40, CD80, CD86, and HLA-DR). In separate experiments, primary untouched human DCs were sequentially MPA-treated, poly I:C-activated, and incubated for 7 days with fluorescently labeled naïve allogeneic T cells. Flow cytometry was then used to quantify allogeneic T cell proliferation.

MAIN RESULTS AND THE ROLE OF CHANCE: Several pharmacologically relevant concentrations of MPA dramatically reduced CD40 and CD80 expression in human primary DCs responding to the immunostimulant poly I:C. In addition, MPA-treated DCs displayed a reduced capacity to promote allogeneic CD4^+ and CD8^+ T cell proliferation. In other DC: T cell co-cultures, the addition of antibody blocking the CD40-CD154 (CD40L) interaction mirrored the decreased T cell proliferation produced by MPA treatment, while addition of recombinant soluble CD154 restored the capacity of MPA-treated DCs to induce T cell proliferation to levels produced by non-MPA-treated controls.

LIMITATIONS, REASON FOR CAUTION: While our results newly reveal that pharmacologically relevant MPA concentrations suppress human DC function in vitro, additional research is needed to learn if DMPA similarly inhibits DC maturation and function in the human female genital tract.

WIDER IMPLICATIONS OF THE FINDINGS: Identification of a mechanism by which MPA impairs human DC activation and function increases the biological plausibility for the relationships currently suspected between DMPA use and enhanced susceptibility to genital tract infection.

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Key words: medroxyprogesterone acetate / dendritic cell activation / costimulatory molecule expression / T cell proliferation
Introduction

Medroxyprogesterone acetate (MPA) is the active molecule in Depo-ProveraTM (DMPA), an injectable, long-acting reversible contraceptive (LARC). Because DMPA is a commonly used LARC in countries with higher prevalences of HIV-1 and other sexually transmitted infections, epidemiological studies indicating that DMPA enhances susceptibility of women to acquisition of these diseases are especially disconcerting. For example, Heffron and colleagues published findings from observational studies in seven African countries showing that women using injectable LARC, including DMPA, were twice as likely to acquire HIV than women not using hormonal contraception (HC) (Heffron et al., 2012). Objections however were quickly raised regarding the reliability of this data, including its low biological plausibility and the high likelihood that results had been confounded by unmeasured differences in condom usage and frequency of unprotected intercourse between the HC users and non-users (Gray, 2012; Hubacher, 2012; Shelton, 2012; van Leeuwen and de Vries, 2012). Moreover, discordant results between other studies exploring links between DMPA and HIV acquisition implied that uncontrolled differences among HC users and non-users were similarly responsible for systematic study biases (Baeten et al., 2007; Kleinschmidt et al., 2007; Morrison et al., 2007, 2010, 2014; Myer et al., 2007; Kumwenda et al., 2008).

The difficulties eradicating systemic bias from such studies, combined with their sizable public health implications, were impetus for developing complementary approaches that more clearly define the connections between HC and sexually transmissible infection (STI) acquisition. One such approach is to delineate HC-mediated alterations in fundamental host defense mechanisms (Murphy et al., 2014), and in our initial efforts using this approach, we found that DMPA treatment of female mice 1 week prior to herpes simplex virus type 1 (HSV-1) mucosal tissue infection significantly reduced dendritic cell (DC) expression of the costimulatory molecules CD40 and CD80. We also found this inhibition of DC activation sharply impaired the expansion of HSV-specific CD4+ and CD8+ T cells and the development of HSV-specific immunologic memory (Vicetti Miguel et al., 2012). As serum MPA concentrations in these mice at the time of infection were comparable to peak serum levels seen in women using DMPA (Mishell, 1996), we hypothesized that pharmacologically relevant MPA concentrations would also suppress human DC activation and function.

We tested this hypothesis in the current study using primary untouched human peripheral blood DC that were sequentially treated with MPA and stimulated with the Toll-like receptor 3 (TLR3) agonist polyinosinic:polycytidylic acid (poly I:C). Analogous to results with the human peripheral blood DC that were sequentially treated (Mishell, 1996), we hypothesized that pharmacologically relevant MPA concentrations would also suppress human DC activation and function.

Materials and Methods

Isolation of primary untouched DC and naïve T cells

Buffy coats were obtained from the Central-Southeast Ohio Region American Red Cross (each buffy coat used for DC or T cell selection contained blood products from a single individual). Peripheral blood mononuclear cells (PBMC) were isolated from these buffy coats by density gradient centrifugation (Ficoll-PaqueTM PLUS, GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Individual PBMC aliquots were placed in cryopreservation media containing 10% dimethyl sulfoxide (DMSO) (Mediatech, Manassas, VA, USA) and 90% fetal bovine serum (FBS) (Atlanta Biologicals, Flowery Branch, GA, USA), and stored in liquid nitrogen. PBMC were subsequently thawed, and primary untouched DCs were isolated via negative immunomagnetic selection using manufacturer’s instructions for the EasySep™ human pan-DC pre-enrichment kit (StemCell Technologies, Vancouver, Canada). After selection, cell viability was routinely ≥85% (as determined by trypan blue exclusion) and DC purity was consistently ≥75% (as determined by flow cytometric analysis) (Supplementary Fig. S1A). To isolate T cells, thawed PBMC were first labeled with CellTrace™ Violet Cell proliferation dye (CTV) (Invitrogen, Eugene, OR, USA), and then T cells were selected using the Pan naïve T cell Isolation Kit (Miltenyi Biotec, San Diego, CA, USA) according to manufacturer’s instructions (viability and purity were ≥95%) (Supplementary Fig. S1B).

Measuring MPA-mediated effects on DC viability and activation molecule expression

In all experiments, MPA solubilized in DMSO (Sigma-Aldrich, St. Louis, MO, USA) was used to form a 2 × 10⁻³ M stock solution. To assess effects of MPA on DC viability, DCs re-suspended in X-VIVO 20 media (Lonza, Walkersville, MD, USA) with 10% AB human serum (Atlanta Biologicals, Flowery Branch, GA, USA) were placed into individual wells of 96-well, round bottom polypropylene plates (2.5 × 10⁵ DCs/well), and incubated for 24 h at 37°C in 5% CO₂ in media plus vehicle or select MPA concentrations (final concentration of DMSO in untreated and MPA-treated wells was <0.001%). Cultures were administered with vehicle alone or poly I:C (1.5 µg/ml), and incubated for an additional 24 h. Cells were harvested and stained with Live/Dead Fixable near-IR (Invitrogen, Eugene, OR, USA) and HLA-DR FITC (G46-6), CD I c PE (B-ly6), CD123 BV421 (9F5) (all BD Biosciences, San Diego, CA, USA) to define myeloid DC (mDC) viability by flow cytometry. To assess effects of MPA on DC activation, other DC cultures were sequentially MPA-treated, poly I:C activated, and stained with Live/Dead Fixable near-IR, an identical mDC antibody mix, and CD80 PE-Cy7 (L307.4), CD40 APC (SC3) and CD86 (FUN-1) (all BD Biosciences). Cells were then treated with Cytotox (BD Biosciences), and examined on a FACS-Canto II Flow Cytometer (BD Biosciences, San Jose, CA, USA). Data were analyzed using FACSdiva (BD Biosciences) and FlowJo (Tree Star, Inc., Ashland OR, USA) software.

Effects of MPA on DC capacity to promote T cell proliferation

In separate studies, primary untouched human DC populations were vehicle- or MPA-treated and poly I:C-activated as described above, then plated (2.5 × 10⁵ DC/well of a 96-well, round bottom polypropylene plate). Allogeneic CTV-labeled naïve T cells were isolated from a different buffy coat, mixed (1 × 10⁵ naïve T cells/well) with DCs, and co-cultures were incubated at 37°C in 5% CO₂ for 7 days (with media replenished every 3 days). Cells were then harvested and stained with Live/Dead Fixable near-IR, CD3 FITC (UCHT1), CD4 PE (RPA-T4), and CD8 PE-Cy7 (RPA-T8)
MPA impairs DC function

MPA suppressed DC expression of CD40 and CD80 induced by poly I:C stimulation

Because MPA concentrations did not affect DC viability, we next explored MPA-mediated effects on DC activation. To do this, primary untouched DCs were isolated from the peripheral blood of eight individuals, and sequentially MPA-treated and poly I:C-activated. Cells were then collected and immunostained to assess expression levels of HLA-DR, CD40, CD80 and CD86 by flow cytometry (representative results shown in Fig. 2A). While analysis of this data revealed no statistically significant differences in DC expression of HLA-DR or CD86 (Fig. 2B and Fig. 2E), DCs showed a slight but statistically significant reduction of CD80 in cultures treated with 0.31 or 0.62 nM of MPA (Fig. 2C). More consistent and profound, however, was the MPA-mediated reduction in DC expression of CD40 (Fig. 2D). This molecule is up-regulated by DC responding to microbial pathogen invasion, and the binding between CD40 and CD154 (CD40L) (expressed on activated T cells) triggers additional DC costimulatory expression and more efficient T cell activation (O’Sullivan and Thomas, 2003). Interestingly, the current findings identifying preferential suppression of CD40 in MPA-treated human DC responding to stimulation with a TLR3 agonist (compared with CD80 and CD86) were entirely congruent with the decreased CD40 expression seen in DCs from MPA-treated mice responding to mucosal HSV-1 infection (Vicetti Miguel et al., 2012).

MPA inhibited DC capacity to stimulate T cell proliferation

As MPA dampened DC response to poly I:C stimulation (particularly DC expression of CD40), we posited that MPA-treated DCs would show a reduced capacity to promote T cell proliferation. To test this hypothesis, primary untouched DCs from the peripheral blood of six individuals were MPA-treated or treated with media alone for 24 h, poly I:C-activated for an additional 24 h, and then cultured with fluorescently labeled naïve allogeneic T cells. Cultures were maintained for 7 days, after which cells were harvested to quantify T cell proliferation levels using flow cytometric analysis (representative results shown in Fig. 3A). As predicted by the inhibitory effects of MPA on DC costimulatory molecule expression, CD40+ and CD80+ T cell proliferation was significantly reduced in cultures containing MPA-treated DC (Fig. 3B and C). Providing evidence that MPA had not directly inhibited the ability of T cells to proliferate, T cell expansion was unaffected when MPA-treated T cells were subsequently stimulated with beads coated with anti-CD3 and anti-CD28 antibodies (Fig. 4). Taken together, these experiments indicated that the MPA-mediated inhibition of DC maturation had been responsible for the impaired T cell proliferation displayed in our DC: T cell co-cultures.

MPA-mediated decrease in CD40 expression reduced DC ability to induce T cell expansion

Since MPA treatment most acutely impacted DC CD40 expression, we hypothesized this effect was responsible for the impaired capacity of MPA-treated DCs to stimulate T cell proliferation. To explore this hypothesis, primary untouched DCs were isolated from the peripheral blood of six individuals, treated with MPA (or media alone) for 24 h, poly I:C-activated for 24 h, and then cultured for 7 days with fluorescently labeled naïve allogeneic T cells. Immediately before adding the labeled T cells, anti-human CD154 antibody was added to a subset of

Statistical analyses

All statistical analyses were performed using Prism 6 software (GraphPad, La Jolla, CA, USA). MPA-mediated effects on DC viability were determined by comparing percentages of live mDCs in vehicle-treated versus MPA-treated cultures (data were normalized by defining vehicle-only cultures to have 100% viability). Values of DC activation marker expression and T cell proliferation were similarly normalized by designating DC marker expression or T cell proliferation in vehicle-only controls to be 100%. In all experiments, normality was tested using the D’Agostino & Pearson omnibus test or by evaluation of the residuals (when experimental sample numbers were <8). One-way ANOVA or Friedman tests with Dunnett’s, Holm-Šídák or Dunn’s post hoc tests were then used for multiple comparisons, depending on data normality (P-values ≤ 0.05 were defined as statistically significant). For comparisons between two groups, the Student’s t-test was used.

Ethical approval

Approval from the Ohio State University Biomedical Sciences Institutional Review Board and the Central-Southeast Ohio Region American Red Cross was received prior to study execution.

Results

MPA treatment did not affect DC viability

We began our investigation of the influence of MPA on human DC activation and function by determining DC viability in untreated and MPA-treated cultures. As detailed in the Materials and Methods, negative immunomagnetic selection was used to obtain primary untouched DCs from the peripheral blood of seven individuals, and these cells were incubated for 24 h with media alone (negative controls) or MPA concentrations ranging between 0.31 and 25 nM (values chosen in consideration of peak [25 nM] and steady-state [2.5 nM] serum concentrations of MPA detected in women using DMPA) (Mishell, 1996). Cultures were then treated with poly I:C (1.5 μg/ml), and incubated for an additional 24 h, after which cells were harvested to assess viability. As our flow cytometric analysis showed, none of the selected MPA concentrations had a significant effect on DC viability (Fig. 1), providing strong indication that our experimental design would be suitable for subsequent assessment of MPA-mediated effects on DC activation and function.

(continued)
the cultures containing untreated (media only) DCs, while rsCD154 was added to a portion of cultures containing MPA-treated DCs at the time of poly I:C activation. As hypothesized, similarly reduced levels of T cell proliferation were seen in cultures containing MPA-treated DCs and cultures containing non-MPA-treated DCs receiving CD154 antibody. Likewise, the addition of rsCD154 restored T cell proliferation in cultures containing MPA-treated DC to levels comparable to those in cultures containing non-MPA-treated DCs (Fig. 5). Such results thus identified reduced CD40 expression as the mechanism by which MPA impairs the capacity of human DCs to promote in vitro T cell proliferation.

**Discussion**

Increasing feminization of the HIV pandemic (Quinn and Overbaugh, 2005) has spurred efforts to identify risk factors modifying the risk of infection in women. While multiple clinical studies imply that DMPA increases the chances of male-to-female sexual transmission, limitations intrinsic to such studies make it unlikely that they alone will establish causality or identify the strength of this association. In particular, it is difficult for clinical studies to eliminate the tight links between HC use and sexual behavior (e.g. more frequent unprotected sex) that commonly confound data interpretation (Warner, 2005) or to identify the exact contraceptive practices of study participants at the time of HIV-1 acquisition (Blish and Baeten, 2011). In addition to feminization, the current HIV pandemic is characterized by a predominance of infections occurring as a result of exposure of the virus to a genital mucosal surface (Cohen et al., 2011). However, as transmission studies indicate that HIV-1 only infrequently disseminates from a genital mucosal surface (Boily et al., 2009), it seems likely there are basic host defense mechanisms that inhibit amplification and systemic spread of the virus (Cohen et al., 2008). Herein, we thus sought to explore biological plausibility for the putative links between DMPA and enhanced susceptibility to HIV-1 infection and MPA-mediated effects with the potential to impair fundamental mechanisms of host defense.
Such efforts were guided by our prior studies in which mice treated with DMPA prior to virus infection of mucosal tissue displayed impaired DC activation, T cell priming, and development of immunologic memory (Vicetti Miguel et al., 2012). These results were compatible with work demonstrating that DCs express progesterone receptors (Butts et al., 2007), and that progesterone treatment of murine bone marrow-derived DC decreased expression of MHC class II molecules, CD80, and CD86, and reduced DC capacity to promote CD4+ T cell

**Figure 2** MPA suppresses the expression of CD40 and CD80 (but not HLA-DR or CD86) in primary human DCs stimulated with the TLR3 agonist poly I:C. Primary untouched DCs were sequentially treated with MPA and poly I:C as described in the Figure 1 legend, and immunostained for flow cytometric analysis of DC marker expression (as detailed in Materials and Methods). (A) Representative contour plots showing CD40 and CD80 expression by mDCs; quadrant numbers indicate percent expression. (B–E) mDC expression of (B) HLA-DR; (C) CD80; (D) CD40; and (E) CD86 (data from five to eight independent experiments normalized and analyzed as defined in Materials and Methods; horizontal bars indicate mean values; *p < 0.05; **p < 0.001).
proliferation (Xu et al., 2011). While our laboratory similarly observed that DMPA treatment of mice reduced CD80 expression in DC isolated from lymph nodes draining viral infection of mucosal tissue (Vicetti Miguel et al., 2012), we saw more profound reduction in DC expression of CD40 (a molecule apparently not examined in the investigation by Xu et al.). Our murine study further identified reduced DC CD40 expression as the mechanism by which MPA impaired in vivo T cell priming and virus-specific T cell expansion (Vicetti Miguel et al., 2012). As such, current demonstration that MPA reduces in vitro CD40 expression levels in primary untouched human DCs responding to poly I:C stimulation, and that this effect impairs the capacity of DCs to induce T cell proliferation confirms and extends our results with a murine DMPA treatment model in which DCs had been activated in vivo by viral mucosal tissue infection.

Although our results differ from earlier reports concluding that progestin-containing compounds do not inhibit human DC expression of CD40 or CD80 (Huck et al., 2005; Segerer et al., 2009), the DCs used in those studies were differentiated in vitro from blood precursors using GM-CSF and IL-4 (compared with the primary untouched human DCs used in the current study). Providing evidence this methodological difference contributed to the discordance of these results, we similarly failed to identify MPA-mediated suppression of CD40 expression when the DCs had been matured in vitro from blood precursors (Supplementary Fig. S2B). We further saw that DC CD40 expression levels prior to poly I:C stimulation were significantly higher when GM-CSF and IL-4 were used to differentiate DC from blood precursors compared with the levels detected in primary untouched DCs isolated by negative immunomagnetic selection (Supplementary Fig. 2A). Such results offer

Figure 3  MPA diminishes the capacity of human primary DCs to promote allogeneic T cell proliferation. Primary untouched human DCs were MPA-treated and poly I:C stimulated as previously described, then cultured with naïve allogeneic T cells labeled with fluorescent dye (CTV). Cultures were maintained for 7 days; then cells were immunostained for flow cytometric analysis of T cell proliferation (as detailed in Materials and Methods). (A) Representative CD4+ T cell proliferation contour plots (numbers signify percentages of proliferating CD3+CD4+ T cells). (B) Proliferation of CD4+ and (C) CD8+ T cells in cultures containing untreated or MPA-treated DCs (data from six independent experiments were normalized and analyzed as detailed in Materials and Methods; horizontal bars indicate mean values; *P < 0.05; **P < 0.01; ***P < 0.001).
a strong indication that the lower levels of CD40 expression in primary untouched DCs facilitated our ability to detect MPA-mediated suppression of DC maturation.

Of particularly importance for increasing biological plausibility of the putative connections between DMPA and STI susceptibility, the dampened DC expression of CD40 and CD80 induced by poly I:C stimulation and the impaired capacity of DCs to promote T cell proliferation seen in our study were produced by pharmacologically relevant concentrations of MPA (0.6–25 nM). Such results are therefore distinct from prior ex-vivo studies in which MPA concentrations that ranged from 600 to 600 000 nM inhibited Type 1 interferon production by human plasmacytoid DCs (pDCs) responding to TLR9 agonists (Hughes et al., 2008), and MPA concentrations ≥100 nM that reduced both secretion of TNF by pDCs stimulated with TLR7/9 agonists (Huijbregts et al., 2013, 2014) and production of Type 2 interferon by human PBMCs stimulated with Bacillus Calmette–Guerin (Kleynhans et al., 2011).

Likewise, while MPA concentrations between 10 and 1000 nM suppressed OVA antigen-specific T cell proliferation (Huijbregts et al., 2013), we saw no MPA-mediated differences in proliferation when T cells were stimulated using beads coated with anti-CD3 and anti-CD28 antibodies. However, in addition to disparate MPA concentrations, effects of MPA on antigen presentation and DC activation may have influenced the results from the earlier investigation, while our study examined the direct effects of MPA on T cell proliferation.

Although our results newly reveal that pharmacologically relevant MPA concentrations inhibit the activation and function of human peripheral blood DCs, further studies are needed to resolve whether MPA and other progestin-based LARC also dampen specific immune responses offering modest protection against HIV spread from mucosal surfaces in the female genital tract. As outcomes from experimental retroviral infection are critically dependent on the speed and extent of virus-specific CD4+ T cell immunity responses (Pike et al., 2009), it is possible that

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**Figure 4** MPA does not directly affect the capacity of T cell to proliferate. CTV-labeled naïve T cells were incubated overnight with vehicle or denoted concentrations of MPA or dexamethasone. Without replacing culture media, T cells were then incubated with beads coated with anti-CD3 and anti-CD28 antibodies. After 5 days, cells were harvested and processed as indicated in the Materials and Methods. (A) Representative contour plots showing CD4+ T cell proliferation contour plots (numbers represent percentages of proliferating CD3+CD4+ T cells). (B and C) Proliferation of CD4+ or CD8+ T cells in T cell-only cultures treated with vehicle, MPA or dexamethasone (data from six independent experiments normalized and analyzed as described in the Materials and Methods; horizontal bars indicate mean values; *P < 0.05; **P < 0.01).
MPA-mediated impairment of DC costimulatory molecule expression and T priming could permit more efficient viral spread. Likewise, DMPA-mediated suppression of DC activation and function has the potential to diminish immunity generated by mucosal vaccination. Amidst growing concern that DMPA is helping to fuel the feminization of the HIV pandemic (Polis and Curtis, 2013), there is therefore a well-defined need for additional research assessing the capacity of this compound to modulate the host responses combating sexually transmitted pathogens in the human female genital tract.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

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Authors’ roles

N.E.Q.C.: participated in study design, data collection, statistical analyses, data interpretation, writing the first manuscript draft, and final approval of manuscript submission. M.G.G.: participated in data collection, data analysis and interpretation, and final approval of manuscript submission. T.L.C.: participated in study concept and design, data analysis and interpretation, manuscript revision, and final approval of manuscript submission. R.D.V.M.: participated in study concept and design, data collection, analysis and interpretation, manuscript revision, and final approval of manuscript submission.

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Conflict of interest

The authors declare they have no conflicts of interest, and that the funding sponsors had no role in the study design, data collection, data analyses, data interpretation, manuscript writing, or in the decision to publish these results.

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